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DEMONSTRATION OF THE AXIAL GRADIENTS BY MEANS OF POTASSIUM PERMANGANATE.

C. M. CHILD.

The existence of graded differences in susceptibility which are essentially quantitative rather than specific, and which show a definite relation to the physiological axes of organisms and parts has already been demonstrated for a large number of plant and animal forms by the various modifications of the susceptibility method, with a wide range of agents and conditions.¹ Additional data on other forms and with other methods, which support and further develop the original conception are as yet unpublished.

The two chief modifications of the susceptibility method, the so-called direct and acclimation methods, are concerned with the effects of different ranges of concentration or intensity of chemical and physical agents. The evidence obtained by these methods indicates that in general the regions or individuals in which the rate of oxidation or metabolism is highest are most susceptible to concentrations or intensities which either kill within a short time or are at least above the limit of acclimation or tolerance of the organism. The results of the acclimation method indicate that the regions or individuals with the highest rate of oxidation or metabolism acclimate or acquire tolerance most rapidly or most completely to concentrations or intensities which are within the limit of acclimation or tolerance. In short, the susceptibility method indicates, first, that both direct susceptibility to concentrations and intensities above the limit of tolerance and ability to acquire tolerance to lower concentrations and intensities of the same agents are associated with differences in the rate of the metabolic reactions of living protoplasm, and second, that the physiological axis *in its simplest form* is essentially a gradient in the rate of fundamental metabolic reactions. It must of course be borne in mind that the chemical reactions

¹ Child: '13, '14, '15a, b, '16a, b, c, d, '17a, b, c, d. Hyman, '16, '17.

themselves constitute only one aspect of such a gradient: associated with them are unquestionable differences in dispersion of colloids, permeability of membranes, enzyme content or activity, electrolytic dissociation and ion content, electrical potential, etc., and the point of view determines, at least to a considerable degree, which factor or group of factors in the complex we regard as fundamental. The essential fact is that these gradients in protoplasmic condition and activity exist and that they are not only characteristic features of physiological axes, but represent the earliest and simplest condition of such axes that we have thus far been able to discover.

The results given by the susceptibility method have been extended and confirmed by various other experimental methods and are not only in complete agreement with, but afford a satisfactory interpretation of a wide range of facts of observation and experiment, such, for example, as axial gradients in protoplasmic structure, in accumulation of yolk and other reserves, in rate of growth and ability to grow at the expense of other parts, in irritability and various other physiological activities.

During the past summer I have made extensive use of potassium permanganate as an agent for demonstrating the physiological gradients in protoplasm and making them directly visible as color gradients. Most of this work was done at the Puget Sound Biological Station at Friday Harbor, Wash., and I am indebted to the director, Dr. T. C. Frye and to the University of Washington for the privileges afforded me.

THE METHOD.

In consequence of its powerful oxidizing action and the stain resulting from its reduction, potassium permanganate has long been used to some extent in histology, and certain investigators have attempted to determine with this and various other reagents the loci of reduction and of oxidation or of low and high oxygen content in cells and tissues. (See for example Golodetz and Unna, '09, P. G. Unna, '11, '13, '15, and various other papers.) The methods and results of these authors have been criticized (Oelze, '14*a, b, c*, Schmidt, '12, Schneider, '14) and their validity defended (Golodetz, '14, Unna, '15), but this controversy does

not affect the well-known fact that $K_2Mn_2O_8$ is readily reduced by protoplasm to MnO_2 which produces a brown coloration of the parts affected. The use of the permanganate for the purpose of demonstrating the gradients in protoplasm is not dependent in any way on Unna's methods and results and does not assume their validity, but is based on the simple assumption that the rate of reduction of permanganate and the consequent coloration of the protoplasm may be expected to show some relation to the physiological condition of the parts concerned, as regards their metabolism, oxidative ability, reducing capacity or avidity or demand for oxygen, in short, to some of the essential factors concerned in the protoplasmic gradients.

In most previous work with permanganate relatively high concentrations have been used, *e. g.*, 1 per cent. by Unna. In such concentrations it is of course a powerful oxidizing agent and extremely toxic, killing many of the lower animals almost instantaneously. In my own work much lower concentrations have been used ranging from $m/1,000$ down to $m/100,000$, *i. e.*, 0.0316 per cent. to 0.000316 per cent.¹ Even in a concentration of $m/1,000$, however, death usually occurs, at least in some parts of the body, within a few minutes. Most of my observations have been made with concentrations of $m/10,000$ or lower, since it has been found that the regional differences in rate of staining appear more clearly in the lower concentration, where the reduction and death occur slowly and progressively, than in the higher, where they are very rapid. In fact, when the organism is killed at once or very rapidly by the permanganate the differences characteristic of the living animal do not appear at all, or are very slight, and when it is killed by some other agent before exposure to permanganate, the differences do not appear.

The results thus far obtained with permanganate have not only confirmed the results of the susceptibility methods, so far as the same forms have been used, but in many cases the great delicacy of the staining reaction has brought out very clearly differences indistinctly or uncertainly shown by the cruder susceptibility methods with lethal concentrations. In fact the

¹ In making up solutions the formula $K_2Mn_2O_8$ with molecular wt. 316 was used. All concentrations are only approximate since solutions were not standardized.

permanganate method makes directly visible gradients such as the gradient in the unfertilized sea-urchin egg, which was not very satisfactorily demonstrated by the direct susceptibility methods (Child, '16*a*), but the existence of which was inferred from the differential inhibition of development resulting from exposure of the unfertilized egg to inhibiting agents (Child, '16*c*). In various cleavage stages, hydroid planulæ, and echinoderm blastulæ and gastrulæ the color gradient is very distinct and uniform, even within the limits of a single blastomere in the earlier stages. In short the method is one of great beauty and delicacy and so entirely simple that it can readily be used for classes or lecture demonstration.

Practically the only precaution to be observed is to provide for uniform distribution and supply of permanganate to all part of the surface of the organism. The permanganate of course gradually disappears from the solution and if the volume of solution is not very large as compared with the protoplasmic surfaces it must be renewed from time to time. Moreover, in the case of organisms lying undisturbed for any considerable time on the bottom of the dish, the surface next the glass always stains less rapidly or less deeply than other regions. These difficulties are readily avoided by slight agitation of the solution at short intervals. Ciliary movement soon ceases in all except very low concentrations of permanganate so that even with motile developmental stages and free swimming protozoa as well as with non-motile forms or stages, frequent agitation of the solution is desirable.

Besides being useful as a staining agent for demonstrating the gradients as color gradients, permanganate can also be used, in at least many cases like KNC and other agents to demonstrate differences in susceptibility as gradients in death and disintegration. Thus far it has been less used in this way than as a staining agent, but it has been found for example that in various ciliate infusoria and early developmental stages of certain forms very low concentrations such as $m/50,000$ bring about death and disintegration before staining occurs. In all such cases observed the disintegration gradient is similar to the staining gradient. In the higher concentrations there is apparently more or less

coagulant or fixing action and so far as my observations go at present, the higher the concentration the less frequent is disintegration.

BRIEF STATEMENT OF RESULTS.

The use of the method thus far has fully justified the assumption on which it was based, viz., that some relation would be found to exist between the rate of reduction of permanganate by living protoplasm as indicated by the rate or intensity of the stain resulting from deposition of MnO_2 and the physiological condition of the protoplasm, particularly its oxidative activity or capacity. It has been found that the regions indicated by other experimental methods and by structural and functional characteristics of the organism as regions of high oxidation rate are most rapidly or most deeply stained in permanganate and also show the highest susceptibility as regards death and disintegration of the protoplasm in the proper concentrations. Moreover, the delicacy of the method indicates clearly the existence of certain minor differences in protoplasmic and metabolic condition, which cruder methods show less clearly or not at all.

Thus far permanganate has been used as a staining agent to demonstrate the gradients in various forms as opportunity offered. These forms include algæ, protozoa, hydroid and medusa forms, eggs and early developmental stages of hydrozoa, a siphonophore, young actinians, polyclad larvae, eggs and various early developmental stages of sea urchins, and ascidian tadpoles. Some observations have also been made on elongated organs such as the tentacles and branchiæ of the polychete, *Amphitrite*. The results as regards the existence of the gradients agree in general very closely with those obtained in other ways, except as regards some minor points shown less clearly or not at all by other methods.

In the algæ examined each active physiological axis of the thallus begins to stain at the apex and the stain progresses basipetally, but in the older regions of the body irregularities appear as with other methods (Child, '16b, '16d, '17a). In monosiphonous branching forms such as *Callithamnium* with elongated cells, the progress of the stain along a single cell can often be observed and in such forms also the gradient in proto-

plasmic disintegration observed with other agents can be clearly seen.

In a study of the determination of polarity by light in the egg of the alga *Fucus vesiculosus*, permanganate and various other agents were used. As is well known, the first steps in *Fucus* development are the cell division with the membrane at right angles to the direction of incident light and the outgrowth of the rhizoid on the side of the egg away from the light. The color gradient with permanganate as well as the susceptibility gradients with KNC and various other agents showed that in the early stages the rhizoid outgrowth stains first and is most susceptible, but that after three to five days a region of rapid staining and high susceptibility begins to arise at the opposite end of the developing plant and this second region becomes and remains the region of highest rate of oxidation as indicated by these methods and forms the apical growing region of the thallus. These data are merely recorded here and will be considered more fully at another time.

Perhaps the most interesting observations among those made on plants concern a diatom of the *Navicula* group. In this form the individuals, bound together by a transparent jelly-like secretion, form an alga-like structure, a pseudothallus, often reaching a length of several centimeters, which shows a definitely directed growth, regular and orderly bifurcation, and in short possesses all the external morphological characteristics of a multiaxiate, branching alga thallus. The appearance of this pseudothallus suggests very clearly that growth and multiplication of the diatoms composing it occur chiefly in the terminal regions of the branches, in fact, it is difficult to account for the orderly axiate habitus on any other basis. In the light of these facts, it is interesting to record that this pseudothallus composed of diatoms shows in permanganate a definite basipetal staining gradient in each branch or axis, for at least several millimeters from the tip, although further basally irregularities appear. A similar gradient in susceptibility has also been observed. In this form then although protoplasmic continuity from diatom to diatom supposedly does not exist, there is a definite, orderly relation, a gradient in physiological condition along each axis, as in axiate

algæ. Some sort of physiological correlation undoubtedly exists, and whatever its nature, it apparently gives rise to the same sort of order and unity as in other axiate forms. It can scarcely be supposed that in this case the correlation is accomplished by chemical substances given off by the individual diatoms, and the only other possibility is a transmissive relation of some sort, probably electrical in character (see R. S. Lillie, '17).

Among the protozoa observations have been made on *Noctiluca*, *Paramecium*, *Stentor* and *Spirostomum*. In *Noctiluca* the first traces of color appear on the surface of the membrane in the mouth region and the color spreads gradually over the membrane. The entoplasm later contracts and disintegrates into droplets before staining, but some slight indications of a basipetal gradient appear in this disintegration. The thickened flagellum of *Noctiluca* shows a well marked basipetal gradient.

In *Stentor* the color gradient in the ectoplasm is essentially the same as the susceptibility gradient already described (Child, '14), *i. e.*, basipetal, but the permanganate indicates a slight degree of "dorso-ventral" difference in that the staining progresses basipetally more rapidly on the oral than on the opposite side of the body. This difference along different meridians of the body has not been observed in susceptibility agents.

Spirostomum shows an ectoplasmic basipetal gradient both in color and in disintegration in permanganate, a short gradient in the opposite direction appearing at the basal end in a varying percentage of individuals. Other agents give essentially similar results. The secondary gradient in this form is very probably associated with the reversibility in the direction of locomotion which this species shows to a very high degree and with stimulation or irritation by the reagent for it apparently occurs only in those individuals which contract strongly and repeatedly in the solution.

In *Paramecium* the first effect of permanganate is, even in very low concentrations, a contraction of the ectoplasm beginning, and most extensive at the anterior end. This results in approach to spherical form, and in the lower concentrations the body often bursts either in the "anal" region or near one of the vacuoles.

In the higher concentrations a slight basipetal color gradient appears, but here again the posterior end commonly also stains almost as rapidly as the anterior, suggesting the existence of a secondary region of high activity there. Earlier work with susceptibility agents indicated a slight basipetal gradient in the majority of individuals, but the results were not as uniform as in many other species (Child, '14). It seems at least possible that certain irregularities in disintegration observed in the earlier work depend in part on the existence of a second region of rather high susceptibility in the posterior part of the body.

Five genera of hydromedusæ, *Phialidium*, *Æquorea*, *Mitrocoma*, *Sarsia* and one undetermined genus, tested repeatedly in permanganate, have shown in all cases a more rapid staining of the ectoderm of the subumbrella, than of the exumbrella. The difference is apparently greater in young than in old animals. These results agree with those of McClendon ('18) on oxygen consumption in the scyphomedusa *Cassiopea xamachana*. He found that the oxygen consumption of the subumbrella, exclusive of the manubrium is more than four times that of the exumbrella in the resting animal, with a still greater difference when pulsations occur. Each medusa tentacle also shows a basipetal color gradient.

In the colonial hydroids, *Bougainvillea*, *Obelia*, *Gonothyria* and one undetermined campanularian genus each hydranth body and each tentacle shows a basipetal color gradient, and with low concentrations a colony gradient also appears, *i. e.*, in a long stem with primary, secondary and perhaps tertiary branches the hydranths and growing tips of the apical region of the whole colony stain more rapidly than those of the basal region and the same differences appear on each of the longer primary branches. This colony gradient is clearly visible to the naked eye when the whole stem with its hydranths is placed in water after staining.

A basipetal color gradient also appears in the stems of the colony, the growing tips always staining much more rapidly and deeply than more basal regions. As regards these stems, however there is of course the possibility that the permanganate may penetrate the thinner perisarc of apical growing regions more readily than the thick perisarc of more basal levels, though, as a matter

of fact, penetration of the perisarc apparently occurs almost at once, and the differences in rate and depth of staining at different levels appear to be very much greater than the differences in perisarc penetration.

The developmental stages of the egg of the hydromedusa, *Phialidium* from the ovarian egg to the hydroid have been examined with permanganate. In the ovarian egg, isolated by teasing, the region of the egg next to the free surface of the gonad stains most rapidly, with a color gradient from this region to the basal egg-pole. This gradient persists during fertilization, cleavage and to the advanced planula stage, the region of most rapid staining being the apical end of the embryo. In the late, elongated planula, just before attachment, a second region of rapid staining arises at what was originally the basal end, the planula attaches itself by its apical end, and the first hydroid arises as a bud from this second region of rapid staining at the basal end. The fact of attachment of the planula by the apical end and the development of the first hydranth from the original basal end has long been known to embryologists, and the MnO_2 color gradients merely serve to give some indication of the physiological conditions concerned. Apparently the first hydranth is physiologically a bud, a process of agamic reproduction in the planula, resulting from physiological isolation at the basal end, a process similar to the development of a second hydranth at the tip of the stolon in *Tubularia* (Child, '15*b*, pp. 91-92) the development of segments in annelids (Hyman, '16 Child, '17*d*), and many other reproductive processes in other forms (Child, '15*b*, Chapter V.). These results on hydrozoa are in agreement with those obtained by other methods, except that as regards unfertilized eggs, cleavage stages and general colony, gradients, the permanganate results are more distinct and definite.

The color gradients have been determined in one species of siphonophore belonging to the family Monophyidae, a form with a single, elongated nectocalyx, from one side of which the stem or cœnosome bearing the groups of zoöids arises. The nectocalyx, which is of course a modified medusa, is morphologically bilaterally symmetrical and shows in permanganate, not only a

more rapid staining of the subumbrella than of the exumbrella like other hydromedusæ, but also a beautiful and striking bilaterality in the staining gradient, the side opposite the origin of the colony stem staining most rapidly, the stem side least rapidly. This difference is also visible with susceptibility methods, but less distinctly. In the groups of zoöids on the stem each zoöid, both nutritive and medusoid, shows a basipetal color gradient and the medusoid zoöids also show a bilateral gradient similar to that of the nectocalyx. In fact, these medusoids along the stem may later become nectocalyces, for each group, consisting of one nutritive, one medusoid zoöid, a group of tentacles and a bract, may become free and develop into a new siphonophore colony. The bilaterality of the color gradient in this form is a very striking feature.

Young actinians including the genus *Peachia* which is parasitic on hydromedusæ during its earlier life-history, and several other undetermined forms showed a very distinct basipetal color gradient in tentacles and body, and with certain concentrations a similar disintegration gradient.

In various embryonic and larval stages of polyclads, a beautiful basipetal color gradient appeared uniformly.

Unfertilized eggs of the sea urchin, *Strongylocentrotus franciscanus* show uniformly a very definite color gradient, although in the absence of definite landmarks it is not absolutely certain that this gradient is basipetal. However, the egg after fertilization and during cleavage does show a distinct basipetal gradient, even in each of the first two blastomeres, and these can be little doubt that this gradient is identical with that of the unfertilized egg. This gradient is also present in blastula and gastrula and is identical with the susceptibility gradient (Child, '16a).

The elongated tentacles and the branched branchial apparatus of the annelid, *Amphitrite* both exhibit very marked basipetal color gradients and in tadpoles of the solitary ascidian, *Corella willmeriana* the growing tail stains most rapidly at the tip with a definite gradient to the base, and the three papillæ of attachment which in this form grow out into temporary stolons during metamorphosis, but are later resorbed, are also regions of rapid staining. On the body of the larva staining progresses from the papillæ more rapidly on the dorsal than on the ventral side.

These miscellaneous data on widely separated groups include the chief results obtained thus far with permanganate. It should perhaps be stated that in every case, except certain hydroids, the siphonophore and actinians at least ten individuals, and in the case of eggs and embryos hundreds or thousands were used, and the experiments were repeated at different times. With some of the hydroids only five or six branching stems from different colonies were used with the same results in all cases. In the case of the siphonophore three colonies were used, and since the results fully confirmed those obtained by other methods, and in each colony the same result could be observed in the different groups of zoöids, larger numbers were considered unnecessary. Of the actinians three to six individuals of each form tested were used, the uniformity of result in all the species making large numbers unnecessary.

DISCUSSION.

The data briefly recorded above are sufficient to show that axial gradients in rapidity of staining with MnO_2 produced by the reduction of permanganate in low concentrations are at least of very wide occurrence in axiate organisms and organs. Moreover, in every case where data have already been obtained by other methods these have been confirmed by the color gradient, and in some cases slight symmetry gradients which were not visible or were indistinct with other methods have been clearly seen.

For various forms, including protozoa, medusa planulæ, echinoderm eggs and embryos and ascidian tadpoles, it has been demonstrated that the color gradient appears only when the living animals are brought into permanganate. When they are first killed without disintegration by some other agent, *e. g.*, mercuric chloride, alcohol, etc., and then brought into permanganate, staining is uniform, except where structural differences are concerned, and the gradient does not appear. Like the susceptibility gradients, the color gradients are thus features of living axiate organisms. This is undoubtedly true for all forms, but its truth has not been proved for all.

Only one further point need be considered here, *viz.*, the question whether the color gradients are merely a result of differences

in rate of penetration of permanganate, in consequence of differences in permeability of different regions of cell or body, or whether they are more directly related to metabolic conditions in the protoplasm. With respect to this question it may be noted first that the color gradient begins to appear as a superficial gradient, apparently resulting from the deposition of MnO_2 on the cell surface. This is particularly evident in single cells, *e. g.*, *Noctiluca*, *Stentor*, eggs and blastomeres, where in optical section it can be seen that no appreciable penetration has occurred when the gradient has already begun to appear. Of course it cannot be denied that in such cases the permanganate has already penetrated a membrane of molecular thickness, but it certainly has not penetrated the visible structural membrane of the cell. In the protozoa the gradient is wholly or almost wholly limited to the ectoplasmic layer, but in eggs it apparently exists to some degree throughout the cytoplasm but appears first superficially. The facts seem at least to indicate that differences in a purely physical permeability of the cell membrane will not account for the color gradient any more than for the susceptibility gradient or the gradients in differential inhibition and acceleration of development.

As regards the question of permeability, however, it is doubtful whether we can isolate it as a purely physical condition from the chemical activity going on in the living protoplasm. Recent investigation indicates more and more clearly that such isolation is impossible. Without going into the matter at length, it is of interest to note that Osterhout has conceived changes in permeability in terms of chemical reaction, that R. S. Lillie in his later papers has emphasized the importance of metabolic reactions in relation to permeability and that recently Crozier ('18*a*, *b*) has concluded that for acids and alkalis the essential factor in stimulation is not an increase in physical permeability by depolarization, but rather a chemical reaction between the agent and some constituent of the receptor cell. And finally, even if we accept a purely physical theory of permeability and assume that the axial gradients in susceptibility to various agents and rate of staining by permanganate represent primarily gradients in permeability, there can be no doubt that such relatively per-

manent, localized and graded differences in permeability in such definite relation to the physiological axes, must be more or less closely associated with differences in metabolic condition, and that in general and within certain physiological limits we may expect to find a higher rate of oxidation associated with a region of higher permeability. Even on this basis then, the axial gradients in susceptibility, color, etc., would serve as indicators of metabolic gradients. But that a purely physical permeability of chemically inert membranes is not the primary factor in this relation is becoming increasingly evident, not merely in the recent modifications in the conception of permeability, but in the facts of susceptibility themselves. For example, differences in permeability of different cells or cell regions along an axis to neutral red and certain other "vital" dyes are usually slight and often inappreciable, although in some cases a distinct permeability gradient can be seen. Nevertheless axial gradients in susceptibility to these agents are very distinct, even within the limits of single cells, whether a gradient in penetration is distinguishable or not. Certainly physical permeability of the cell membrane is not the primary factor in such susceptibility gradients. Again, in the processes of acclimation and recovery that region of an axis which is most susceptible to the agent in high concentration or intensity undergoes acclimation most rapidly or completely in low concentrations or intensities, or recovers most rapidly and completely after temporary exposure within certain limits. Obviously the axial gradients of acclimation and recovery are very directly dependent upon metabolic rate in protoplasm and purely physical differences in permeability are of minor importance.

In the light of all the facts, then, I believe we are justified in concluding as regards the color gradients resulting from the protoplasmic reduction of permanganate; first, that they are indications of fundamental quantitative differences in the physiological condition of protoplasm, second, that such condition is very intimately related to the rate of oxidation, third, that the quantitative graded differences thus indicated represent the physiological axis in its simplest form.

SUMMARY.

The axial gradients in many plants and animals can be very clearly and beautifully demonstrated as color gradients by the use of low concentrations of $K_2Mn_2O_8$, the color being due to the reduction of the permanganate to MnO_2 by protoplasm.

Axial gradients have been demonstrated by this method in all axiate organisms thus far examined including several algæ, a diatom pseudothallus, four species of protozoa, various hydroids, hydromedusæ and their developmental stages, a siphonophore, actinians, developmental stages of polyclads and echinoderms, axiate appendages of polychetes, and ascidian tadpoles.

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